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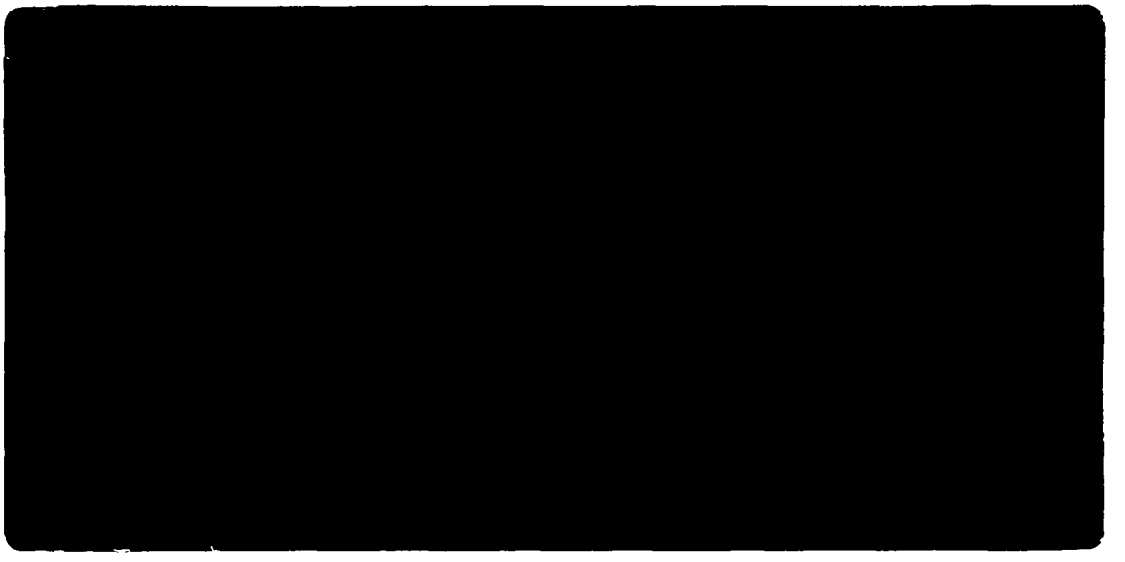

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DEVELOPMENTS IN GENETIC MANIPULATION OF WOODY PLANT FIBER AND ENERGY TRAITS

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INTRODUCTION

The Institute of Paper Science and Technology (IPST) has conducted research in cell and tissue culture for over two decades. First efforts dealt with propagation of aspen (Populus tremuloides), and IPST scientists succeeded in producing plants from culture in the 1960s (Winton 1968). Since the 1970s, however, major emphasis has been on somatic embryogenesis of commercially important softwoods. Somatic embryogenesis has been obtained in several species at IPST, most recently in loblolly pine (Pinus taeda) (Becwar et al., 1990; Uddin et al., 1990) and Douglas-fir (Pseudotsuga menziesii) (Nagmani and Dinus, in press, 1991). Further development of the process may yield a rapid, inexpensive means for mass clonal propagation of elite genotypes, produced either by classical or molecular genetics.

Hardwood cell and tissue culture research was renewed recently. Focus of the hardwood program centers on producing useful variants of eastern cottonwood (Populus deltoides) via genetic transformation and somaclonal variation/selection. Traits of interest include herbicide tolerance and enhanced auxin synthesis. Both are considered important to efficient fiber and energy production. Herbicide tolerance promises substantially lower plantation establishment costs and increased growth. Enhanced auxin synthesis is expected to influence fiber numbers and/or dimensions (Klee et al., 1987). Increased fiber numbers, of course, equate to greater productivity. Altered dimensions, e.g., longer fibers with thinner walls, could raise paper quality and product variety as well as ease conversion to energy via fermentation or pyrolysis (Dinus et al., 1990). Also, ability to alter, at will, auxin status in cultures and/or plants will permit investigation of mechanisms underlying fiber formation.

A general overview of current IPST hardwood research and brief summary of recent, even if preliminary, results follows.

PROGRAM DESCRIPTION AND STATUS

The program consists of several steps, which if executed successfully should yield useful variants. Individual steps are defined in Figure 1, although somewhat arbitrarily, to promote clarity of description. Briefly, development of useful variants requires ready access to clean explants from plants of interest; reliable methods for establishing, maintaining, and multiplying cultures; efficient means for effecting genetic transformation and selecting somaclonal variants;

reproducible protocols for regenerating plants from culture; and techniques for confirming genetic change, stability, and utility.

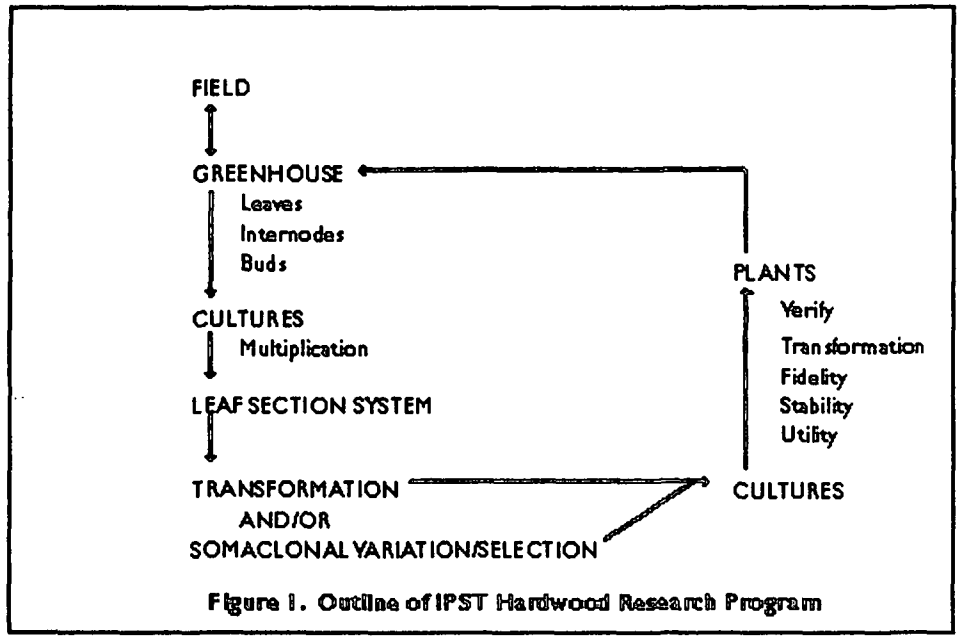

Figure 1. Outline of IPST Hardwood Research Program

PLANT MATERIALS

Research currently involves six cottonwood genotypes (clones); numbers are kept low to facilitate method development. Two model clones, C175 and K417, known for ease of manipulation in culture are being used along with four elite clones. C175 (Dr. S.G. Ernst, University of Nebraska Lincoln, Origin = Wabasha Co., MN) has proven especially easy to manipulate and is used to develop methods for all program steps, including genetic transformation. The other model, K417 (Dr. C. S. Prakash, Tuskegee University), is being moved through various steps as quickly as possible. K417 was incorporated because of its more southerly origin (Fulton Co., KY) and suitability for eventual testing on sites of immediate interest -- bottomlands along the southern Mississippi and western Columbia Rivers.

Elite clones St 66, 70, 72, and 75 (Issaquena Co., MS) were supplied by Dr. B. J. Stanton, James River Corp., Camas, WA. St 66, 70, and 75 (Table 1) were chosen for site adaptability, superior volume productivity, average or better specific gravity, and variable alpha-cellulose and lignin contents (Olson et al., 1985). St 72 was acquired for site adaptability and superior productivity. Plans call for extension of methods, as developed with model clones, to elite clones. Thus, St 66 and 75 have been established in culture and are being readied for tests of responsiveness to multiplication and transformation protocols developed with C175 and K417.

Table 1. Attributes of three elite cottonwood clones chosen for research on genetic transformation and/or somaclonal variation and selection[1].

Clone No.	Volume (cu m)	Specific Gravity	a-cellulose (%)
St 75	0.0694	0.32	53.7
St 66	0.0674	0.34	51.3
St 70	0.0665	0.32	49.5
Test Mean[2]	0.0490	0.33	51.1

[1] Adapted from Olson et al., 1985.
[2] A total of 75 clones were tested in the trial.

Whether received as cultures or dormant nonrooted cuttings, all clones are represented in the IPST greenhouse by at least three ramets. Individual ramets are maintained in 2-gal pots containing a commercial potting mix of peat, perlite, and vermiculite. Water is provided as needed, and fertilizer (Peters 20-20-20 with micronutrients) is applied weekly. These measures plus day/night temperature and photoperiodic regimes suited to year-round growth are used to ensure availability of leaves, internodes, and other explants regardless of season. Commercially available fungicides and insecticides, two each, are applied alternately across weeks to prevent and/or remedy pest problems. Clean greenhouse environments are essential to minimize fungal and bacterial contamination. Ramets are hedged every four to six weeks to maintain modest size, facilitate pesticide application, and maximize explant numbers.

CULTURE ESTABLISHMENT, MAINTENANCE, AND MULTIPLICATION

Two approaches are used to establish cultures. Developing internodes and leaves are explant types of choice. Internodes are used to establish shoot cultures and thereby supply explants for other steps. Leaf sections are used to multiply cultures and for transformation and somaclonal variation/selection trials.

Internode Culture: Culture establishment with internodes generally follows protocols developed by Coleman and Ernst (1989, 1990a). They induced shoot formation by exposure to Modified Woody Plant Medium containing 0.5 mg/l zeatin (Z) either with or without prior incubation on

the same medium containing 0.5 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D). Incubation for various lengths of time on 2,4-D, depending on genotype, stimulates callus formation, with subsequent Z exposure inducing shoots.

We repeated several treatments used by Coleman and Ernst (1990a) in an effort to discern responsiveness of the St clones and to secure materials for establishment of cultures. Observed were percentages of explants forming shoots (regardless of shoot length or extent of development) nine weeks after start of culture and percentages of explants elongating shoots at 13 weeks.

Results for C175 generally agree with those of Coleman and Ernst (1990a). Shoots formed on fair to large percentages of internode explants following brief incubation on 2,4-D and subsequent exposure to Z (Table 2). Somewhat lower numbers formed in response to longer 2,4-D exposures, incubation with Z alone, and on the control or basal medium. Elongation occurred most frequently following exposure to 2,4-D.

Overall treatment and clone effects were statistically significant ($p = 0.05$). K417 proved most responsive and gave the highest percentages of explants with shoots suitable for subsequent use. Though not differing from one another, St clones proved significantly less responsive than K417 and C175. Elite genotypes responded in terms of shoot formation, but performed poorly as regards elongation. Useful numbers of elongated shoots, however, were obtained.

Regardless of variable, treatment by clone interactions were also significant; treatments useful for one clone were not always good for others. This finding underscores importance of genotype and the need, at least for the foreseeable future, to tailor protocols to individual clones.

Effects of internode position or developmental status were also significant, confirming earlier work by Douglas (1984) with a Populus hybrid and perhaps providing a means for raising efficiency with even seemingly recalcitrant clones. Importance of internode position varied with clone. Explants of the most responsive clones, K417 and C175, produced elongated shoots regardless of internode position. For the less responsive St clones, however, elongated shoots were produced only by the fifth through ninth internodes from the tip, and the seventh through ninth were several fold more responsive than others. First and second internodes were not used, as per Coleman and Ernst (1989).

In sum, this approach appears applicable to the IPST program, and we are seeking improved results by testing additional 2,4-D exposure lengths, evaluating higher Z levels, and preferentially using the most responsive internodes. Such refinements should raise efficiency with the aforementioned clones and permit extension to a wider array. We are also attempting to multiply St cultures using proliferation and elongation protocols and various modifications thereof, advanced by Coleman and Ernst (1990b).

Table 2. Mean percentages of cottonwood internode explants forming (FS) and elongating (ES) shoots after incubation with 2,4-D (0.5 mg/l) for various lengths of time before exposure to Z (0.5 mg/l). Data were taken after nine and 13 weeks of culture.

Clone No.	Variable FS(±SE) ES(±SE)	Clone Means	Control (Basal Medium)	Treatments: Days of Incubation on 2,4-D Before Exposure to Z 0	1	4	8
K417	FS	74.2a[1]	31.0(15.4)[2]	86.9(5.1)	92.5(3.4)	86.9(8.7)	66.7(13.6)
	ES	70.0a	23.0(0.0)	79.8(6.3)	86.5(5.1)	83.6(4.4)	69.4(10.9)
C175	FS	84.4a	75.0(25.0)	80.6(7.9)	94.4(3.5)	79.8(10.8)	86.7(8.2)
	ES	38.1b	0.0(0.0)	13.5(6.7)	50.6(11.8)	56.7(16.4)	47.5(20.6)
St 66	FS	23.0b	0.0(0.0)	30.6(7.6)	38.4(21.5)	21.9(6.5)	20.7(12.0)
	ES	8.8c	0.0(0.0)	7.5(5.0)	0.0(0.0)	18.6(8.0)	13.6(9.4)
St 70	FS	19.5bc	16.7(16.7)	13.8(7.1)	20.2(8.7)	30.6(17.4)	12.5(12.5)
	ES	6.2c	0.0(0.0)	0.0(0.0)	23.2(9.6)	5.6(5.6)	0.0(0.0)
St 72	FS	8.2c	16.7(16.7)	12.5(6.0)	8.3(8.3)	0.0(0.0)	2.8(2.8)
	ES	0.6c	0.0(0.0)	2.8(2.8)	0.0(0.0)	0.0(0.0)	0.0(0.0)
St 75	FS	19.6bc	50.0(28.9)	30.1(10.5)	25.6(9.5)	4.8(3.0)	2.8(2.8)
	ES	4.4c	0.0(0.0)	4.8(4.8)	12.7(8.1)	2.4(2.4)	0.0(0.0)
Treatment Means:	FS	38.1	26.3c(3)	42.4ab	47.9a	37.8abc	31.4bc
	ES	21.9	5.0c	18.1b	30.8a	27.8a	22.8ab

[1] Clone means represent mean percentages of explants forming (FS) or elongating (ES) shoots across all treatments for individual clones after nine and 13 weeks in culture, respectively. Means followed by different letters differ significantly from other means for same variable according to Duncan's multiple range test at $p = 0.05$.

[2] Means and standard error of means for individual clone X treatment combinations.

[3] Treatment means represent mean percentages of explants forming (FS) and

elongating (ES) shoots across all clones after incubation with 2,4-D (0.5 mg/l) for various lengths of time before exposure to Z (0.5 mg/l). Means followed by different letters differ significantly from other means for same variable according to Duncan's multiple range test at p = 0.05.

Leaf Section System: Genetic transformation via Agrobacterium tumefaciens (At) has been accomplished with leaf disks in various plant species, including Populus hybrids (Fillatti et al., 1987; Sellmer and McCown 1989), and such systems should obviate need for protoplast or more complicated systems (McCormick et al., 1986). Transformation of leaf disks, followed by development and rooting of adventitious shoots, is considered a straightforward and efficient approach (Horsch et al., 1985).

Several methods are available for obtaining organogenesis in Populus species and hybrids (e.g., Lee-Stadelmann et al., 1989), but cottonwood has proven more difficult to manipulate (Sellmer et al., 1989). Earlier work by Prakash and Thielges (1989) showed that adventitious shoots can be regenerated from leaf callus of cottonwood, but the protocol involves numerous steps and may yield somaclonal variants as a result of the extensive intervening callus phase. Against this background, we endeavored to develop a simple, efficient leaf section protocol for regenerating adventitious shoots, well-suited for transformation, and incurring less callus formation.

Second, third, and fourth leaves of C175 were collected and cut into 5 X 10 mm rectangular pieces, each containing a portion of the midvein and wounded on all sides. Sections were placed horizontally on modified Woody Plant Medium (MWPM) (Prakash and Thielges, 1989) or DKW-C Medium (McGranahan et al., 1987) containing various growth regulator types and concentrations.

Shoot initiation and elongation occurred without excessive callus production. N^6-benzylaminopurine (BAP) and Naphthaleneacetic Acid (NAA) applied together (1.0 or 2.0 µM each) and Thidiazuron (TDZ) alone (0.1 µM) produced the largest numbers of shoots per explant. When retested, best treatments produced similar responses regardless of basal media (Table 3).

Dark culture (3 weeks) was necessary for shoot induction, but elongation was best in the light (3-6 weeks). More than 90 percent of elongated shoots rooted in 2-4 weeks on Woody Plant Medium, supplemented with 0.1-0.5 µM Indolebutyric Acid (IBA). Normal, vigorous plants were recovered and transferred to greenhouse conditions 12 to 14 weeks after explants were placed in culture. In a parallel student project, elongated shoots formed on 30 percent of explants, and an average of eight rooted shoots were obtained from explants forming shoots. Efforts are being made to extend application to more clones.

Table 3. Number of shoots (Mean +/- Stan. Dev.) initiated and elongating per explant after culture for six weeks on MWPM or DKW-C basal media supplemented with BAP and NAA or TDZ. Explant Number per treatment = 60.

Basal Medium	Growth Regulators (μM)	Shoots / Explant (No.)
MWPM	1.0 BAP & NAA	2.4 +/- 1.1
DKW-C	1.0 BAP & NAA	3.9 +/- 1.4
"	2.0 " " "	2.2 +/- 1.1
DKW-C	0.1 TDZ	2.3 +/- 1.2

GENETIC TRANSFORMATION

Our first attempts to effect transformation involve enhanced auxin synthesis and Kanamycin (K) resistance. Assays were performed to determine sensitivity of C175 leaf sections to the antibiotics, K and Carbenicillin (C). Using K resistance as a selective marker requires adding K to cultures in quantities sufficient to eliminate nontransformed materials while permitting putative transformants to form shoots. In addition, C levels were needed that would eliminate At without inhibiting shoot production. Several screening trials showed that C175 leaf sections were quite sensitive to K; concentrations of 20 mg/l or more proved lethal. Shoot production was not affected by C levels as high as 500 mg/l.

For transformation trials, C175 leaf sections were cocultivated for two days with At Strain pMON518, containing genes for enhanced auxin synthesis and K resistance coupled with a constitutive promoter (CaMV 19S). The At and genetic construct were secured for research purposes from Monsanto Corp. Cultures were grown in darkness on DKW-C medium containing 1.0 μM BAP and 1.0 μM NAA. Sections were then transferred to the same medium and growth regulators, supplemented with 150 mg/l K and 500 mg/l C, and cultured in dark for an additional 19 days. After the 3-week shoot induction period, surviving cultures were moved to light conditions for shoot elongation.

One percent of the leaf sections survived, producing either callus or shoot-like structures. Though lower than desired, this level of putative transformation is in line with the literature (e.g., Fillatti et al., 1987). Plants have not yet been recovered, but all cultures continue to grow on the

same medium and growth regulators, both with and without K. Cultures have phenotypes similar to nontransformed ones given high exogenous levels of auxin. These lines of evidence suggest successful transformation for both K resistance and auxin synthesis. Efforts to stimulate shoot production by altering exogenous growth regulator regimes continue. With further culture growth, transformation will be confirmed by Southern blotting and comparing auxin levels of transformed and nontransformed cultures. To avoid problems caused by the constitutive promoter; i.e., apparent constant expression of the enhanced auxin synthesis genes, future trials will be done with a construct containing the same genes and a heat shock promoter, also obtained for research purposes from Monsanto Corp. In addition, we are seeking cooperation with other laboratories that possess promoters specific to cambium.

SOMACLONAL VARIATION/SELECTION

As presently constituted, research on this front is focused on development of glyphosate tolerant cells and plants of cottonwood. Rationales are that this approach may proceed faster than that using genetic transformation and that variants would not be subject to the regulatory constraints likely to affect genetically transformed materials.

Glyphosate, a broad spectrum herbicide, was selected because of its safety and utility in cottonwood management regimes. The herbicide inhibits EPSP-synthase, a key enzyme in aromatic amino acid synthesis (Mazur and Falco, 1989) by bacteria and plants but not animals.

Since glyphosate retards aromatic amino synthesis, effects should be both more severe and more easily detected in rapidly growing cells. Thus, we chose to work with cell suspensions since they grow rapidly and form large populations for selection but require little time, effort, and space.

Several workers have produced plant cell lines tolerant of glyphosate. Carrot (Dacus) and Catharenthus rosus cell lines have been produced with 52- and 60-fold increases in tolerance, respectively (Nafziger et al., 1984; Cresswell et al., 1988). Similar increases in petunia have been attributed to additional copies of the EPSP-synthase gene (Shah et al., 1986). Michler and Haissig (1988) obtained tolerant variants in Populus hybrids following exposure of leaf discs to various glyphosate doses.

Presently, research at IPST is being done on three aspects of somaclonal variation/selection. The first concerns establishment of cell suspension systems, the second involves development of methods to regenerate plants from suspensions, and the last concerns selection of herbicide tolerant cells and subsequent recovery of intact plants.

Establishment of Cell Suspensions: First, work concentrated on securing friable callus for establishment of cell suspensions. Leaf sections of C175 were used as explants, and various levels of 2,4-D and BAP as well as 2,4-D and Kinetin were tested in a factorial arrangement on MS Medium (Murashige and Skoog, 1962). Callus formed in response to all combinations of

2,4-D and BAP, but the only Kinetin treatment yielding callus was that containing 2.0 mg/l 2,4-D and 1.0 mg/l Kinetin. Subjective evaluations deemed the latter treatment best on the basis of growth rate and ease of establishing suspension cultures.

Calli derived from the aforementioned experiments were used to develop protocols for establishing and maintaining suspensions. Based on earlier exploratory work, several levels of 2,4-D and BAP were evaluated in liquid MS Medium. Cultures were grown in the dark and shaken at 125 rpm. Fine suspensions formed quickly and growth was rapid; best results were obtained with 1.0 mg/l 2,4-D and 0.1 mg/l BAP. Suspensions judged best by visual checks of appearance and growth were those derived from callus produced in response to 2.0 mg/l 2,4-D in combination with 1.0 mg/l Kinetin.

In later work, growth of C175 cell suspensions was assessed more objectively in replicated trials. Measures included were settled cell volume, fresh weight, dry weight, protein content, and cell count. Cultures were established in 500 ml of MS Medium with 1.0 mg/l 2,4-D and 0.1 mg/l BAP and grown in the dark at 22°C and shaken at 125 rpm. Fresh and dry weight determinations provided the simplest and most reliable measures.

Regardless of measurement method, C175 cell suspensions consistently exhibited a generation time of three days. The lag phase lasted 5-7 days, and exponential growth occurred from day 7 through 19. Cultures entered the stationary phase after 19 days, and cultures older than 28 days were not reliable for use in starting new suspensions.

Regeneration of Plants: As a first step toward regenerating plants, small volumes of C175 suspension (10 ml or less) were plated on solid MS Medium supplemented with three levels each of 2,4-D and BAP. Cultures were grown in the dark, and small calli formed in 3-4 weeks. Developing clumps periodically were consolidated into larger masses to hasten growth and save time. Usable calli were obtained with 1.0 or 2.0 mg/l 2,4-D and 0.05 mg/l BAP.

For shoot induction, 20 C175 calli (0.5-1.0 g), obtained as described above, were transferred to DKW-C Medium supplemented with 1 μM BAP and 1 μM NAA. Cultures were grown under low light intensity and subcultured biweekly. Rudimentary shoots formed in 3-4 weeks, with some forming as late as the 10th and 15th subculture. Elongation of individual shoots required several weeks after first appearance. An average of 1.5 shoots (0.5 cm and longer) were harvested from each clump and rooted via the protocol for shoots from leaf sections. Derived plants appear normal and are growing well in the greenhouse. Results also indicated that calli from suspensions maintained for more than several subcultures will not form shoots. Until refinements are made, new suspensions must be established with plants produced from each preceding suspension in order to maintain the cycle and permit selection across succeeding populations.

Selection of Herbicide Tolerant Cells: To develop tolerant cell lines, C175 suspensions were established, grown for seven days to the early exponential phase, and then challenged with glyphosate, 0, 8, 16, 32, 64, 128, 256, and 512 μM.

After 7, 14, and 21 days of growth, samples were withdrawn to test viability and isolate tolerant cells and/or cell clumps. One ml samples were extracted and stained with tetrazolium chloride to determine viability. Living cells and tissues stain pink upon exposure to this reagent (Reinert and Yeomann, 1982). In addition, 0.5 ml samples were withdrawn and plated on a solid, herbicide-free version of the suspension medium and grown in the dark for 21 days to monitor relative size and growth potential of surviving cell populations.

Staining with tetrazolium chloride revealed mixtures of living and dead cells in samples from cultures given herbicide doses of 64 μM or greater. At concentrations above 64 μM, large proportions did not stain pink, indicating significant, but limited, mortality. At the highest concentrations (256 and 512 μM), over 90% of cells failed to stain and were presumed dead.

Growth on solid medium was not retarded by challenge with glyphosate concentrations of 128 μM or less. Substantial growth retardation, however, was noted at higher concentrations. Some cells, however, were alive and began forming calli within several days. Calli from suspensions exposed to the 256 and 512 μM concentrations have been maintained for futher research. Later trials with six of the same concentrations, including the control, and several higher ones, 1024, 2048, 4096, and 8192 μM, lead to isolation of cell lines tolerant of 512 and 8192 μM glyphosate. These and the two lines noted above will be used in rechallenge experiments and to determine biochemical and molecular basis of tolerance.

SUMMARY AND CONCLUSIONS

The foregoing describes current hardwood cell and tissue culture research at IPST. The program, individual steps, and results to date are detailed to the extent possible at this time. Goals, stated in practical terms, call for production of useful cottonwood variants via genetic transformation and/or somaclonal variation/selection. Traits of interest include, but are not limited to, herbicide tolerance and increased auxin production. Improvements could lead to lower costs of fiber and energy production and raise quality of wood and fiber. Reliable protocols for individual steps, of course, are valuable in their own right. Enhanced auxin synthesis could provide means for studying mechanisms controlling fiber formation and enlargement. Finally, somaclonal variation could yield improvements other than herbicide tolerance, e.g., altered lignin types and amounts, increased cellulose content, unusual fiber properties, pest resistance, or drought tolerance.

Much progress has occurred despite the short history of the program. Model and elite clones have been acquired, and appropriate greenhouse cultural practices have been implemented. Workable systems for establishing and maintaining cultures have been developed. Much work remains to be done on these aspects, particularly as regards application to elite clones. Nevertheless, materials have been placed in culture and are being maintained as sources of clean explants for work on other steps. The leaf section system yields plants at rates on par with systems developed elsewhere and is suitable for genetic transformation. Putative transformants with enhanced auxin synthesis have been obtained, and acquisition of an improved genetic

construct should hasten confirmation of transformation and recovery of transformants. Efforts on extension to other clones are underway. Cell suspensions were developed and characterized. Preliminary results indicate that plants can be regenerated, at least for one model clone. Evidence to date further indicates that suspensions can be used to produce herbicide tolerant cell lines. Further work is needed to recover plants and confirm tolerance. Also, much work will be required to extend the technology to commercially valuable clones.

This last issue is of extreme importance. Refinement of protocols is essential to ensure that each yields reproducible results, is efficient, and works well for a variety of genotypes. Model clones are useful, but our work indicates that one protocol seldom is optimal for several or more genotypes. The tendency for elite clones to be recalcitrant must be overcome. Also, plants regenerated in vitro, transformed or not, must perform in greenhouse and field comparable to cuttings or seedlings now in use. Physiological or genetic aberrations can occur in culture, especially in plants derived from tissues other than preformed meristems. Performance of derived plants must therefore be ascertained quickly and definitively. This need not mean that methods described above will not work or will fail to yield useful plants. Rather, it underscores the need to check performance constantly and to develop means for early and inexpensive testing. Related work on such methods, hopefully, will permit early and accurate checks of fidelity and stability.

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